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1 **Adaptive modulation of antibiotic resistance through intragenomic**
2 **coevolution**

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11

12 **Bacteria gain antibiotic resistance genes by horizontal acquisition of**
13 **mobile genetic elements (MGE) from other lineages. Newly acquired**
14 **MGEs are often poorly adapted causing intragenomic conflicts, resolved**
15 **by compensatory adaptation of the chromosome, the MGE or reciprocal**
16 **coadaptation. The footprints of such intragenomic coevolution are**
17 **present in bacterial genomes, suggesting an important role promoting**
18 **genomic integration of horizontally acquired genes, but direct**
19 **experimental evidence of the process is limited. Here we show adaptive**
20 **modulation of tetracycline resistance via intragenomic coevolution**
21 **between *Escherichia coli* and the multi-drug resistant (MDR) plasmid**
22 **RK2. Tetracycline treatments, including monotherapy or combination**
23 **therapies with ampicillin, favoured *de novo* chromosomal resistance**
24 **mutations coupled with mutations on RK2 impairing the plasmid-**
25 **encoded tetracycline efflux-pump. These mutations together provided**
26 **increased tetracycline resistance at reduced cost. Additionally, the**
27 **chromosomal resistance mutations conferred cross-resistance to**
28 **chloramphenicol. Reciprocal coadaptation was not observed under**
29 **ampicillin-only or no antibiotic selection. Intragenomic coevolution can**
30 **create genomes comprised of multiple replicons that together provide**
31 **high-level, low-cost resistance, but the resulting co-dependence may**
32 **limit the spread of coadapted MGEs to other lineages.**

33

34

35 Horizontal gene transfer (HGT) is a fundamental process in bacterial evolution
36 that accelerates adaptation by sharing ecologically important accessory traits
37 between lineages¹. These accessory traits are themselves frequently located
38 on semi-autonomous mobile genetic elements (MGE), such as conjugative
39 plasmids, that encode genes for their own replication, partition and horizontal
40 transfer^{2,3}. Conjugative multidrug resistance (MDR) plasmids, encoding
41 antibiotic resistance genes (ARG) against multiple classes of antibiotics, are
42 of particular clinical concern since they allow instantaneous acquisition of
43 MDR phenotypes and thus potentiate the rapid emergence of MDR bacterial
44 pathogens^{4,5}. Newly acquired conjugative plasmids are often costly since the
45 plasmid is unlikely to be well adapted to the new genetic background, causing
46 intragenomic conflict⁶. The cost of plasmid carriage is likely to arise due to the
47 metabolic burden of maintaining, transcribing and translating plasmid genes⁷,
48 as well as via disruption of cellular homeostasis caused by gene regulatory
49 interference between chromosomal and plasmid regulators^{8,9}, and cytotoxic
50 effects of plasmid gene products⁶.

51

52 An important route to resolving this intragenomic conflict is compensatory
53 evolution to ameliorate the cost of plasmid acquisition¹⁰. Experimental
54 evolution suggests that compensatory evolution can arise via mutations
55 located on either the chromosome or the plasmid, or via intragenomic
56 coevolution involving both plasmid and chromosome mutations^{8,9,11,12}.
57 Comparative genomics suggests a key role for compensatory evolution in
58 natural bacterial populations, potentially stabilising MDR plasmids within
59 lineages and thus allowing the evolutionary emergence by HGT of MDR

60 pathogens¹³. A key outstanding question is how the mode of compensatory
61 evolution following MDR plasmid acquisition varies with antibiotic treatment.
62 Here we experimentally evolved *Escherichia coli* MG1655 carrying the MDR
63 plasmid RK2 (encoding tetracycline and ampicillin resistance genes) under a
64 range of antibiotic treatment regimes including no antibiotic, mono- and
65 combination-therapies of tetracycline and ampicillin. Following 530
66 generations of selection we quantified evolved changes in antibiotic
67 resistance and fitness, and used genome sequencing to determine the
68 genetic bases of the observed adaptation.

69

70 **Results**

71 Thirty independent isogenic populations of *E. coli* MG1655 carrying the MDR
72 plasmid RK2¹⁴, which encodes resistances to tetracycline (TET) and ampicillin
73 (AMP), were experimentally evolved for ~530 generations (80 days), under
74 five antibiotic treatments (six independently evolving lines per treatment): no
75 antibiotic (N), AMP (A), TET (T), AMP plus TET (AT), and 24 hour cycling
76 between AMP and TET (A/T) (see methods). Plasmids remained at high
77 frequency in all populations for the duration of the selection experiment.
78 Plasmid-free segregants were only observed at very low frequency in two of
79 the six populations from treatment N (Supplementary Fig. 1), whereas
80 transposition of resistance genes from RK2 onto the host's chromosome was
81 never observed.

82

83 To test for changes in antibiotic resistance profiles following evolution we first
84 determined the minimum inhibitory concentration (MIC) of evolved lineages to

85 TET and AMP. The susceptibility of the evolved strains to antibiotics differed
86 between treatments (Fig. 1a). We observed a four-fold increase in TET MIC in
87 evolved strains from the T and AT treatments and a small increase in lineages
88 that had evolved under the cycling A/T treatment compared with the ancestral
89 MG1655 with ancestral RK2 (Anc-RK2), whereas evolved strains from
90 treatments N and A showed no change in tetracycline MIC (ANOVA, $F_{5,30} =$
91 6.103, $p < 0.001$; Post-hoc Tukey Tests, Anc-RK2:T $p < 0.001$, Anc-RK2:AT p
92 < 0.01 , Anc-RK2:N $p = 0.525$, Anc-RK2:A $p = 0.783$). By contrast, we
93 observed no change in resistance to AMP in any treatment (ANOVA, $F_{5,30} =$
94 1.212, $p = 0.327$), possibly due to a lower relative selection pressure imposed
95 by the concentration of AMP used in the experiment compared to the
96 concentration of TET¹⁵. Interestingly, TET selection led to the evolution of
97 bacteria that were cross-resistant to chloramphenicol (CML), although the
98 extent of the evolved cross-resistance varied between treatments (ANOVA,
99 $F_{5,30} = 24.25$, $p < 0.001$); with CML MIC increasing 8-, 4-, and 2-fold in T, AT,
100 A/T treatments, respectively. Consistent with CML cross-resistance being a
101 correlated response to TET selection, evolved strains from both the N and A
102 treatments remained equally sensitive to CML as the ancestral MG1655(RK2)
103 (Post-hoc Tukey Tests, Anc-RK2:N $p = 0.975$, Anc-RK2:A $p = 0.993$). Thus
104 whereas T and AT treatments, and to a lesser extent the cycling A/T
105 treatment, led to the evolution increased TET resistance and cross-resistance
106 to CML, evolved lineages from the N and A treatments showed no change in
107 their resistance profile.
108

109 To examine the genetic bases of evolved changes in resistance we next
110 obtained whole genome sequences for one randomly selected clone per
111 population. Excluding hypermutators, evolved clones had acquired between 2
112 and 11 mutations, located exclusively on the chromosome in non-TET
113 treatments (C, N, A), and on both the chromosome and plasmid in the
114 treatments including TET (T, AT, A/T) (Supplementary Fig. 2, Supplementary
115 Table 1). Of all the observed mutations 13.2% were synonymous and 19%
116 were intergenic, the remaining non-synonymous mutations (67.8%) comprised
117 missense mutations (42.8%), frameshifts (10.6%), insertion sequences (5.6%)
118 and gene deletions (5.4%), and these were analysed further. While the
119 variance in the number of non-synonymous mutations did not differ between
120 treatments (Analysis of multivariate homogeneity of group variances excluding
121 hypermutators $F_{5,26} = 1.8617$, $p = 0.1358$), the loci affected by non-
122 synonymous mutations did vary between treatments (Permutational ANOVA,
123 permutation test: $F_{5,26} = 2.5231$, $p < 0.01$, Bonferroni corrected). Clones that
124 had evolved under TET selection (T, AT, A/T) had significantly different sets
125 of non-synonymous mutations compared to evolved clones from the other
126 treatments (C, N, A) (permutation test: $F_{1,30} = 6.9463$, $p < 0.01$, Bonferroni
127 corrected), with a larger genetic distance between TET and non-TET
128 treatments than within these treatment groups (Fig. 2a). Thus TET-selected
129 lineages followed an evolutionary trajectory distinct from non-TET-selected
130 lineages, leading to mutations on both the chromosome and the plasmid,
131 which suggest that TET selection favoured bacteria-plasmid coadaptation.
132

Strikingly parallel mutations were observed between independent replicate populations both within and between TET-containing treatments (Fig. 2b). Highly parallel mutations are likely to represent adaptive evolution at these loci, and because mutations at these loci were not observed in the populations from the N and A treatments, these mutations were likely to be TET-specific adaptations. Mutations in the chromosomal genes *ompF* (16 out of 18 clones), and *ychH* (16 out of 18 clones) showed strong locus-level parallelism within all three TET-containing treatments. Mutations in *ompF*, encoding a major non-specific diffusion porin¹⁶, were all predicted loss-of-function mutations, including the insertion of IS elements, frameshifts or premature stop codons. The loss of OmpF in *E. coli* reduces membrane permeability, including to antibiotics, and consequently is known to increase resistance to a wide spectrum of antibiotics¹⁷ including TET and CML^{18,19}. Deletion of *ompF* (*E. coli* K-12 $\Delta ompF$ JW0912²⁰) significantly increased resistance to TET without the RK2 ($t_{9.09} = 4.2836$, $p < 0.01$), and further increased TET resistance when carrying RK2 (Two-way ANOVA Interaction $F_{1,20} = 14.724$, $p < 0.01$; Supplementary Fig. 4a). Parallel loss of function mutations (IS elements and frameshifts) in *ychH* were observed across all the TET treatments. YchH is a hypothetical stress-induced inner membrane protein^{21,22}, but deletion of *ychH* (*E. coli* K-12 $\Delta ychH$ JW1196²⁰) did not significantly increase the resistance to TET with or without the plasmid (Supplementary Fig. 4b), suggesting that this general stress response may not be required under TET selection and is consequently selected against.

157 Mutations in several loci observed in the T and AT treatments were not
158 present in the cycling A/T treatment. These included mutations in both *acrR*
159 (10 out of 12 clones) and *adhE* (9 out of 12 clones). Mutations in *adhE* were
160 extensively parallel at the nucleotide level, with 8 clones from independent
161 populations all having the same missense SNP in the ethanol dehydrogenase
162 domain²³. The phenotypic significance of these mutations is unclear due to
163 the multiple roles assigned to this protein, including multiple metabolic
164 pathways²⁴, but intriguingly the AdhE protein is known to exhibit binding
165 activity to the 30S ribosome²⁵, the primary TET target. The *acrR* gene
166 encodes a repressor of AcrAB multidrug efflux pump²⁶, the majority of
167 mutations in *acrR* are predicted loss of function mutations, with IS elements
168 and frameshifts observed in evolved strains. The deletion of *acrR* results in
169 the overexpression of *acrAB* leading to MDR phenotypes^{27,28}. Deletion of *acrR*
170 (*E. coli* K-12 Δ *acrR* JW0453²⁰) alone did not significantly increase resistance
171 to TET ($t_{9.32} = -0.591$, $p = 0.339$), but when combined with the RK2 plasmid
172 did allow significantly increased growth in TET ($t_{6.4} = 3.665$, $p < 0.01$,
173 Supplementary Fig. 4c). These findings are consistent with the higher TET
174 resistance of evolved clones from the T and AT treatments versus the A/T
175 treatment (Fig. 1a) and reflect overall weaker TET selection under the A/T
176 cycling compared to the T and AT treatments where TET selection was
177 constant. Interestingly, stronger TET selection appeared to constrain evolution
178 at chromosomal loci not involved in resistance. For example, we observed
179 highly parallel loss of function mutation in the flagellum operon in the A, N and
180 A/T treatments, but only rarely observed mutations at these loci in T and AT
181 treatments. Loss of the flagellar motility is a commonly observed adaptation of

182 *E. coli* to growth in liquid media²⁹ and this may have been impeded by clonal
183 interference or negative epistasis with chromosomal resistance mutations in
184 populations under strong TET selection. Consistent with this, whereas
185 evolved clones from the N and A treatments increased in fitness relative to the
186 plasmid-free ancestor in antibiotic-free media, such fitness gains were not
187 observed in evolved clones from the TET-containing treatments
188 (Supplementary Fig. 5).

189

190 To confirm that TET selection had led to the evolution of chromosomal
191 resistance we next cured evolved strains of their plasmids and quantified
192 resistance³⁰. Evolved strains carrying putative chromosomal resistance
193 mutations displayed increased TET (ANOVA, $F_{5,30} = 42.63$, $p < 0.001$), AMP
194 (ANOVA, $F_{5,30} = 12.55$, $p < 0.001$) and CML (ANOVA, $F_{5,30} = 35.88$, $p <$
195 0.001) resistance (Fig. 1b). Across all tested antibiotics, evolved clones
196 carrying both *ompF* and *acrR* mutations had significantly increased resistance
197 compared to the ancestral MG1655 (Post-hoc Tukey Tests, all $p < 0.05$),
198 whereas cured evolved strains without either of these mutations (i.e. from the
199 N and A treatments) did not (Post-hoc Tukey Tests, all $p > 0.05$). Interestingly,
200 cured evolved clones from the cycling A/T treatment that carried only
201 mutations in *ompF* but not in *acrR* showed marginally increased resistance to
202 both TET and CML, but no detectable increase in AMP resistance, relative to
203 MG1655. Thus TET selection favoured the *de novo* evolution of chromosomal
204 resistance despite pre-existing plasmid-encoded TET resistance, and these
205 chromosomal resistance mutations are responsible for the observed cross-
206 resistance to CML.

207

208 We observed parallel mutations on the plasmid exclusively in evolved clones
209 from the TET-containing treatments (T, AT, and A/T). These mutations
210 occurred in *tetA/tetR* (18 out of 18 clones; *tetA*: 13, *tetR*: 2, both: 3, Fig. 2b)
211 which encode the tetracycline-specific efflux pump. The expression *tetA* is
212 tightly regulated by the repressor *tetR* in the absence of tetracycline^{31,32}.
213 Mutations in *tetA* were dispersed throughout the gene, affecting the protein's
214 transmembrane, periplasmic and cytoplasmic domains³³. Three of the five
215 mutations observed in *tetR* are in direct contact with or in close proximity to
216 the tetracycline binding pocket³⁴, while the other two mutations are located in
217 the central scaffolding of the protein, suggesting that they are likely to
218 interfere with activity of the *tetR* repressor. Evolved plasmids carrying
219 mutations in *tetA* or *tetR* displayed reduced resistance to TET in the ancestral
220 MG1655 background compared to ancestral RK2 (Fig. 1d, ANOVA, $F_{5,30} =$
221 4.586, $p < 0.01$). Consistent with reduced efficacy of plasmid-encoded
222 resistance in evolved lineages with *tetA/tetR* mutations, when we replaced the
223 evolved plasmid with ancestral RK2, this led to increased TET resistance
224 (ANOVA, $F_{5,30} = 71.86$, $p < 0.001$, Anc-RK2:T,AT,A/T all $p < 0.05$).

225

226 Our data suggest that evolved strains from TET-containing treatments
227 adapted their resistance to TET by acquisition of weak chromosomal
228 resistance mutations in combination with mutations that reduced the efficacy
229 of the plasmid-encoded TET efflux pump. To understand the evolutionary
230 benefits of this counterintuitive dual resistance strategy we first compared the
231 effect of chromosomal background (evolved or ancestral) and plasmid

232 genotype (evolved or ancestral) on growth in the presence of 10 µg/ml TET
233 (i.e., the concentration used in our selection experiment). The evolved
234 chromosomal background carrying resistance mutations displayed a
235 significantly shortened lag phase compared to the ancestral chromosomal
236 background, irrespective of the plasmid genotype (Supplementary Fig. 6;
237 ANOVA, $F_{3,56} = 76.92$, $p < 0.001$; Post-hoc Tukey Tests, Evolved
238 Host:Ancestral Host all $p < 0.001$). This suggests that chromosomal
239 resistances reducing membrane permeability to antibiotics allowed evolved
240 strains to start growing faster in the presence of TET.

241

242 Whereas evolved bacteria grew equally well with evolved or ancestral
243 plasmids, ancestral bacteria displayed impaired growth with evolved
244 compared to ancestral plasmids (Supplementary Fig. 6; Max OD, $W = 93$, $p <$
245 0.01). This is consistent with the mutations in *tetA/tetR* reducing resistance
246 but importantly confirms that this reduction is not evident when in combination
247 with the chromosomal resistance mutations, which appear to compensate for
248 the reduced efficacy of the plasmid-encoded efflux pump.

249

250 We next competed evolved bacteria with either the evolved or ancestral
251 plasmid against the ancestral MG1665(RK2) to compare the costs of carrying
252 each plasmid genotype. The ancestral plasmid displayed a significantly higher
253 cost than the evolved plasmid in the evolved chromosomal background
254 (Supplementary Fig. 7, $t_{25,71} = -2.287$, $p < 0.05$). This suggests that the
255 mutations to *tetR/tetA* ameliorate the cost of plasmid carriage but at the price
256 of reduced efficacy of TET efflux. This is consistent with previous studies

257 showing a high cost of expressing the specific tetracycline efflux pump³⁵.
258 Taken together with the growth data, this suggests that although mutations to
259 *tetA/tetR* reduce growth under tetracycline in the ancestral chromosomal
260 background, they have minimal effect on resistance in the evolved
261 chromosomal background due to the reduced membrane permeability and
262 additional efflux systems expressed in the evolved chromosomal background
263 carrying mutations in *ompF* and *acrR*, leading to high resistance and a
264 lowered cost of plasmid carriage. This suggests that the chromosomal
265 resistance mutations must have been gained prior to the mutations in the
266 plasmid-encoded tetracycline efflux pump. To test this, for one population
267 (AT2) we tracked the frequency over time of an observed IS-insertion in *ompF*
268 by PCR and then determined by sequencing when these genotypes acquired
269 mutations in the *tetA/tetR* genes. Consistent with the hypothesised order of
270 mutations, the IS-insertion in *ompF* was first detected at transfer 8 and had
271 swept to fixation by transfer 32, whereas mutations in *tetA/tetR* were not
272 observed in this *ompF*::IS background until transfer 32 (supplementary figure
273 8).

274

275 **Discussion**

276 Our current model of bacterial evolution suggests that horizontal acquisition of
277 ARGs accelerates resistance evolution by providing bacteria with ready-made
278 resistance mechanisms, bypassing the requirement for rare *de-novo*
279 mutations¹. However, recent population genomic data suggesting that
280 lineages independently acquire and then subsequently coevolve with MDR
281 plasmids^{13,36,37} imply a more dynamic evolutionary process. Consistent with

282 this, here we show here that gaining an ARG can be just the starting point in
283 the evolution of resistance and, due to the costs of expressing horizontally
284 acquired ARGs, does not preclude subsequent *de novo* evolution of
285 chromosomal resistance. Evolved strains from TET-containing treatments
286 gained chromosomal resistance mutations reducing membrane permeability
287 and enhancing efflux of TET and providing cross-resistance to other
288 antibiotics, shortening lag phase in the presence of TET. These mutations
289 also reduced the need for a fully operational plasmid-encoded tetracycline
290 efflux pump, expression of which is highly costly³⁵, allowing plasmid mutations
291 in the TET efflux pump and its regulator which reduced the cost of plasmid-
292 encoded resistance. A consequence of this intragenomic coevolution is that
293 the increased TET resistance of evolved strains from T, AT and A/T
294 treatments required the action of both the chromosomal- and plasmid-
295 encoded resistances, which together acted multiplicatively. Thus intragenomic
296 coevolution can lead to the evolution of bacterial genomes comprised of co-
297 dependent replicons, limiting the potential for onward transmission of the
298 plasmid due to the weaker resistance it now encodes in other lineages.

299

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435

436

437

438 **Methods**

439 **Strains, culture conditions and evolution experiment**

440 *E. coli* MG1655 chromosomally labelled with GFP at the *attB* lambda
441 attachment site was used in the evolution experiments. Isogenic *E. coli*
442 MG1655-mCherry was used as a reference strain in competition and
443 conjugation rate experiments. Both *E. coli* strains were provided by the Van
444 Der Woude lab (University of York). The RK2 plasmid was introduced to the
445 strains through conjugation from *E. coli* MV10 provided by the Thomas lab
446 (University of Birmingham). All cultures were grown in Oxiod® Nutrient Broth
447 (NB) at 37°C 5 ml in 50 ml microcosms shaken at 180 rpm. Independent
448 selection lines were founded by 30 independent single colonies of *E. coli*
449 MG1655-GFP harbouring RK2. These were grown overnight in non-selective
450 conditions and split into the 5 antibiotic treatments, no antibiotic selection, 100
451 µg/ml ampicillin, 10 µg/ml tetracycline, 100 µg/ml ampicillin plus 10 µg/ml
452 tetracycline, and 24 hour cycling between 100 µg/ml ampicillin and 10 µg/ml
453 tetracycline, with 6 replicate populations per treatment. In parallel, 6

454 independent *E. coli* MG1655-GFP colonies were picked for control treatments
455 and grown under no selection. Selection lines were established by transferring
456 50 µl of saturated overnight culture into 5ml of selective media. These
457 populations were maintained through transfer of 1% of the population into
458 fresh media and antibiotics every 24 hours for 80 transfers, resulting in ~6.64
459 generations per day, totalling ~530 bacterial generations. For the cycling
460 treatment 3 populations were initiated with 100µg/ml ampicillin and 3
461 populations were initiated with 10µg/ml tetracycline. Culture density (OD₆₀₀)
462 was recorded every 24 hours. Plasmid prevalence was measure at the start
463 and end of the selection experiment by screening 20 randomly picked
464 colonies from each population using multiplex primers specific to RK2
465 replication origin (Fw: ctcatctgtcaacgccgc, Rv: aaccggctatgtcgtgct), β-
466 lactamase (Fw: ataactacgatacgggagggc, Rv: acatttccgtgtcgccctta), and
467 tetracycline efflux pump (Fw: tgggttctctatatcgggcg, Rv: tgggcgagtgatgcagaat).
468 These primers allowed for the detection of plasmid loss and transposition of
469 resistances onto the chromosome. One end point clone was randomly
470 selected from each population for phenotypic typing, curing, calculation of
471 MICs and sequencing. Every eight transfers throughout the experiment 500 µl
472 samples of whole populations were collected and stored in 25% glycerol at -
473 80°C. Whole populations were also plated out on non-selective media, 20
474 individual clones were then randomly selected, sub-cultured for a further 24
475 hours in non-selective media, and stored in 25% glycerol in 96 well plates.

476

477 **Relative Fitness**

478 The relative fitness of the evolved plasmid bearing versus ancestral plasmid
 479 free strain was estimated by direct competition, with six replicate strains per-
 480 treatment. The competitions were initiated with 50 µl of 1:1 mixtures of
 481 plasmid-bearing evolved strain and plasmid-bearing ancestral strain marked
 482 with mCherry from overnight cultures in 5ml of non-selective NB media. The
 483 relative fitness of the evolved strains was calculated by gaining exact viable
 484 cell counts at 0 hours and 24 hours, strains were distinguished through
 485 detection of fluorescent markers using Zeis Stereo Lumar v12 microscope.
 486 The relative fitness of plasmid-bearing bacteria was calculated as a ratio of
 487 Malthusian parameters³⁸:

$$W_{evo} = \frac{\ln\left(\frac{N_{final,evo}}{N_{init,evo}}\right)}{\ln\left(\frac{N_{final,anc}}{N_{init,anc}}\right)}$$

488
 489 Fitness effects due to different markers was determined by competing plasmid
 490 free MG1655-GFP with plasmid free MG1655-mCherry, the relative fitness of
 491 MG1655-GFP was not significantly difference from 1 ($t_5=0.015584$, $p=0.9882$)
 492 showing that there is no significant difference between the two marker strains.

493
 494 Relative fitness of evolved strains harbouring evolved plasmid or evolved
 495 strains harbouring ancestral plasmid versus ancestral plasmid bearing cells
 496 was estimated using the same method as above, with eighteen replicate
 497 strains per competition, but grown in 100 µl cultures in a 96 well plate, 37°C
 498 shaken at 600 rpm, 3 mm orbital radius, inoculated to an initial dilution of
 499 1:500. Again no fitness effect of markers was observed ($t_5=-0.2795$, $p=0.791$).

500

501 **Curing RK2 from evolved strains**

502 Evolved strains were cured using the pCURE curing system³⁰. The anti-incP-1
503 cassette (RK2 *oriV*, *parD*, *korA*, and *incC* genes) from pCURE11 was ligated
504 into the pLAZ2 chloramphenicol resistant vector that contains the *sacB* gene
505 allowing counter selection for plasmid free segregants. The resultant plasmid
506 was transformed into chemically competent evolved strains and selected for
507 using Cml 12.5 µg/ml. Single colony transformants were re-streaked on to
508 Cml 12.5 µg/ml plates and Cml 12.5 µg/ml + 5% sucrose. Sucrose sensitive
509 colonies were checked by PCR for the presence of the curing plasmid (Fw:
510 aagttttggtgactgcgctc, Rv: caaagacgatgtggtagccg) and absence of RK2 β-
511 lactamase and *tetA* (primers as above). Successfully cured clones were
512 cultured for 24 hours in non-selective media to allow segregation of the curing
513 plasmid; sergeants were selected on antibiotic free, 5% sucrose plates. To
514 confirm loss of both plasmids sucrose resistant colonies were check for
515 sensitivity to chloramphenicol, ampicillin, and tetracycline, as well as PCR
516 using primers mentioned above. Both the ancestral strain harbouring RK2 and
517 ancestral plasmid free strains under went the curing process and were used
518 as a comparison to cured evolved strains to control for curing process.
519 Ancestral RK2 was introduced into the cured evolved strains, and evolved
520 RK2 was introduced into the plasmid free ancestor though conjugation. Again,
521 to control for the curing and conjugation steps, ancestral RK2 was conjugated
522 into cured ancestral strains and used for comparison. Saturated overnight
523 cultures of donor plasmid containing strains and recipient plasmid free strains
524 were mixed 1:1, and 50 µl was used to inoculate 5ml NB. The mixed cultures
525 were grown for 24 hours and plated out on to 100 µg/ml ampicillin to select for

526 transconjugants. Transconjugants were confirmed by fluoresces and PCR
527 screening for RK2 plasmid.

528

529 **MIC**

530 To measure minimal inhibitory concentrations, six replicate cultures per-
531 treatment were grown overnight until stationary phase in 5 ml NB, the
532 saturated cultures were then sub-cultured 50 µl into 5 ml fresh NB and grown
533 to an OD₆₀₀ of 0.5. These were then diluted into 96-well plates containing a
534 log₂ serial dilution of antibiotic (AMP, TET or CML) to an initial density of
535 5×10⁵ CFU/ml. 100 µl cultures were grown for 24 hours 37°C shaken at 600
536 rpm, 3 mm orbital radius. OD₆₀₀ was measured after 24 hours.

537

538 **Growth Curves**

539 Six replicate saturated overnight cultures per-treatment were sub-cultured to
540 an OD₆₀₀ of 0.5, and used to inoculate 100 µl NB supplemented with 10 µg/ml
541 TET per well in 96-well plates at a final dilution of 1:1000. Plates were grown
542 at 37°C with shaking at 300 rpm, 3 mm orbital radius for 24 hours, OD₆₀₀ was
543 measured every 16 minutes by Tecan infinite M200 Pro plate reader. Growth
544 rates were calculated as the maximum slope of log₂ transformed OD₆₀₀
545 covering four time points (~1 hour of growth), lag phase was calculated to end
546 when growth rate reached 10% of the maximum achieved growth rate.

547

548 **Genome sequencing and analysis**

549 Whole genomes were extracted from each evolved population's clone as well
550 as the ancestral strain and ancestral strain harbouring the RK2 plasmid using

the DNeasy Blood and Tissue extraction kit (Qiagen). The total DNA was sequenced by MicrobesNG (<http://www.microbesng.uk>), which is supported by the BBSRC (grant number BB/L024209/1), using Illumina MiSeq. Reads were mapped to *E. coli* MG1655 K-12 genome (GenBank accession U00096.3) and RK2 (GenBank accession BN000925.1) reference using BWA-MEM³⁹. Single nucleotide variants and small indel events were detected using GATK UnifiedGenotyper⁴⁰ and SnpEff⁴¹, insertion sequences were identified using custom scripts and Integrative Genomics Viewer⁴², and large genome-wide structural variants were detected using BreakDancer⁴³. Mutations that were present in the ancestral clones were excluded, resulting in a set of mutations that were acquired during the selection experiment.

562

563 **Tracking mutations**

Populations that did not show a hypermutator phenotype, had insertion sequences within *ompF*, and mutations in the tetracycline resistance genes on the plasmid, from the constant TET treatments (T and AT treatments) were selected for further analysis to gain an understanding of the mutational timeline during the selection experiment. Insertion sequences within *ompF* were identified within whole populations of T4, AT2, AT3 and AT5 by PCR of the *ompF* gene (Fw: ACTTCAGACCAGTAGCCAC, Rv: GCGCAATATTCTGGCAGTGA). A short product of 716 bp indicated no insertion sequence, a long product of 1484 bp indicated IS1 and a long product of 1911 bp indicated IS5. Whole population PCR indicated that *ompF*::IS mutants had swept into the population by transfer 40 for populations T4, AT2 and AT5, and transfer 48 in population AT3. Frequency of *ompF* insertion

576 sequences were calculated by PCR of 20 clones from transfers 8, 16, 24, 32,
577 and 40. Tetracycline resistance genes (*tetA* and *tetR*) from clones containing
578 *ompF*::IS mutations from population AT2, transfers 8, 16, 24, and 32 were
579 then Sanger sequenced to determine if *ompF* mutations arise before *tetAR*
580 mutations (*tetA*: Fw: GGCTGCAACTTTGTCATG, Rv:
581 TTCCAACCGCACTCCTAG, Internal1: ACAGCGCCTTTCCTTTG, Internal2:
582 AAGGCAAGCAGGATGTAG; *tetR*: Fw: TCTGACGCGGTGGAAAG, Rv:
583 ACGCGCGGATTCTTTATC, Internal1: GAGCCTGTTCAACGGTG, Internal2:
584 TCTGACGACACGCAAAC).

585

586 **Statistical analysis**

587 To test if the mutations observed within each treatment had significantly
588 different variances a multivariate homogeneity of groups variances test was
589 conducted⁴⁴. The binary presence or absence of a variant at each allele was
590 use to calculate a Euclidean distance matrix between each population. This
591 was used to test for homogeneity of variances between treatments using
592 *betadisper* {vegan 2.4-0}. The variances between treatments were
593 significantly different, with hypermutators significantly affecting within-group
594 variation. These clones were removed from further analysis as significant
595 differences in within-group variance can lead to falsely significant results
596 when testing for differences between groups⁴⁵. Permutational Multivariate
597 Analysis of Variance was used to calculate whether different evolutionary
598 treatments resulted in different sets of mutations^{45,46}. Using the Euclidean
599 distance matrix with hypermutators removed, the significance of within- and
600 between-group distances was calculated using *adonis2* {vegan 2.4-0}. The

601 data was partitioned into different groups, multiple testing was corrected for
602 using Bonferroni correction. Neighbour Joining phylogeny was constructed
603 using the binary presence or absence table with hypermutators removed.
604 Tree estimation and bootstrap support was conducted using *ape-package*
605 {ape 4.0}. Significant difference between two related samples was calculated
606 using two sided, two-sample t-test. Shapiro-Wilk test was conducted to check
607 for normality, when normality could not be assumed a non-parametric
608 Wilcoxon signed-rank test was used. Differences among treatments growth
609 under antibiotic selection were calculated by ANOVA of the integral of the
610 resistance profiles, with subsequent Tukey multiple comparison of means. All
611 statistical analysis was conducted in R (version 3.2.3).

612

613 **Data Availability**

614 The sequence data supporting the findings of this study are available at the
615 European Nucleotide Archive, accession: PRJEB20735. All other data in this
616 study is available at Figshare data depository
617 (<https://doi.org/10.6084/m9.figshare.5092225.v1>). Custom code used to map
618 possible IS elements are available online
619 (https://github.com/mbottery/Co_Evo_IS_Analysis).

620

621

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631

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633 the experiments and analysed the data. All authors contributed towards the
634 design of the study and wrote the manuscript.

635

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637

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640

641 **Figure 1 | Resistance profiles of evolved plasmids and hosts.** Growth of
642 **a**, evolved MG1655 strains with evolved RK2 plasmids **b**, evolved MG1655
643 strains cured of evolved RK2 plasmids **c**, evolved MG1655 strains with
644 ancestral RK2 plasmid and **d**, ancestral MG1655 clones with evolved RK2
645 plasmids in the presence of tetracycline, ampicillin or chloramphenicol in
646 comparison to ancestral MG1655. Points represent means of one clone from
647 each of the six independent treatment populations, with SEM error bars.
648 Dashed grey and black lines show the resistance profiles of plasmid free and
649 plasmid containing ancestral strains respectively. Dashed lines in evolved
650 host cured of plasmid plots (**c**) show ancestral MG1655 and ancestral

651 MG1655(RK2) after curing process. Dashed lines in ancestral host evolved
652 plasmid plots (d) show ancestral MG1655 and ancestral MG1655(RK2) which
653 had under gone curing with ancestral RK2 subsequently reintroduced.
654 Vertical dashed lines in AMP and TET resistance profiles show the
655 concentrations of AMP (100 µg/ml) or TET (10 µg/ml) used in the selection
656 experiment.

657

658 **Figure 2 | Mutations show treatment specific parallelism. a**, An unrooted
659 neighbour joining phylogeny of end-point evolved clones. The distance matrix
660 was constructed from the binary presence or absence of variants at each
661 gene relative to the ancestral strain; hypermutators were excluded from the
662 analysis. Scale bar represents number of gene variants; percentage bootstrap
663 support is shown at the branches, B=1000, values below 0.3 are omitted. Blue
664 branches represent clone isolated from TET treatments. **b**, Mutations
665 observed in evolved clones (excluding hypermutators) across treatment.
666 Rings represent *E. coli* chromosomes or RK2 plasmids. Dots represent
667 mutations, the size of the dots represent the number of mutations at the same
668 loci across independent replicate populations. Plots of individual treatments
669 are in Supplementary Fig. 3.



